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USE OF A MODIFIED FLUORESCENT *IN SITU* HYBRIDIZATION PROCEDURE TO IMPROVE THE IDENTIFICATION OF *STREPTOCOCCUS PNEUMONIAE* IN BLOOD CULTURES

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Streptococcus pneumoniae is an important causative agent for bacteremia. Fluorescent *in situ* hybridization (FISH) is a helpful molecular technique for the rapid identification of *S. pneumoniae* in positive blood cultures. There are many reports concerning the application of an enzymatic treatment with lysozyme in the FISH procedure for partial cell wall digestion of *S. pneumoniae*. However, this study was aimed to test the FISH procedure without enzymatic treatment for the identification of *S. pneumoniae* in blood culture specimens. Seventy-seven positive blood culture specimens containing Gram-positive cocci were examined by both the conventional laboratory methods and FISH. Detection of *S. pneumoniae* was performed by two FISH procedures: one procedure was performed with an enzymatic treatment step and the other one was done without enzymatic treatment. In addition, the specimens were tested by the FISH procedure with enzymatic treatment to detect *Streptococcus pyogenes* and *Enterococcus*. The specificity of FISH in comparison with conventional culture methods was 100%. The sensitivity of the FISH procedure with enzymatic treatment for the detection of *S. pneumoniae* was 90%, whereas, the sensitivity of the FISH procedure without enzymatic treatment was 100%. In fact, by omission of enzymatic treatment, detection of *S. pneumoniae* was improved in 6 specimens. The results of the FISH and culture methods for the detection of *S. pyogenes* and *Enterococcus* were compatible. Altogether, FISH procedure without enzymatic treatment step seems to improve the detection of *S. pneumoniae* in some cases. Thus, for successful detection of *S. pneumoniae,* we suggest the application of both FISH procedures (the procedure with enzy-

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matic treatment and the procedure without enzymatic treatment) for each blood culture specimen.

Keywords: *Streptococcus pneumoniae,* fluorescent *in situ* hybridization, FISH, lysozyme

Introduction

Bloodstream infections are main causes of morbidity and mortality. Gram-positive bacteria cause 65% of bloodstream infections in United States hospitals [1]. Streptococci and enterococci that are Gram-positive organisms, are among the important etiologic agents for bloodstream infections [1, 2]. *Streptococcus pneumoniae* (pneumococcus) is a major causative agent for bacteremia in both children and adults [3]. Approximately one-fifth of patients admitted to hospitals because of a pneumococcal pneumonia, have bacteremia [4]. The mortality of pneumococcal bacteremia can be about 20% [5].

Conventional methods for the identification of etiologic agents of bacteremia are time-consuming and results can be achieved 1 to 2 days (or may be longer for fastidious organisms) after detection of microbial growth in blood culture systems [6, 7]. This delay may impede appropriate therapy of patients [6]. Therefore, rapid identification of causative pathogen is essential for early selection of proper antimicrobial drugs and improving the prognosis of the patients with bacteremia. Also, based on this strategy, unnecessary treatment of contaminants can be avoided [7].

Fluorescent *in situ* hybridization (FISH) with rRNA-targeted fluorescently labeled probes, is a useful molecular technique for the rapid identification of microorganisms in positive blood cultures [7–10]. In the FISH procedure, the enzymatic treatment is performed to open the peptidoglycan layer of the Gram-positive bacteria. Thus, oligonucleotide probes can penetrate into the bacterial cell. In previous studies, enzymatic treatment with lysozyme has been performed on *S. pneumoniae* during examination of blood cultures or other specimens [7, 8, 11, 12]. During a series of examinations in our laboratory, a question was raised as to whether the enzymatic treatment step is always necessary for *S. pneumoniae*. Therefore, the objective of this study was to test the FISH procedure without an enzymatic treatment step for the identification of *S. pneumoniae* in blood culture specimens.

Materials and Methods

Blood culture specimens

This project was approved by the Ethical Committee of Bushehr University of Medical Sciences. Seventy-seven positive blood culture specimens containing Gram-positive cocci were collected and examined by using both cultivation and FISH methods to detect *S. pneumoniae, S. pyogenes,* and genus *Enterococcus*.

A volume of 200 µl of each blood culture specimen was subcultured on blood agar plates. The plates were incubated at 35ºC in a candle jar [13–15] for the isolation of the bacteria investigated in this study. After incubation, the suspicious colonies on blood agar were considered and the identification of aforementioned organisms was performed by using conventional laboratory methods [16].

For FISH, 500 µl of each blood culture sample was fixed by adding an equal volume of absolute ethanol (Merck, Darmstadt, Germany).

Reference strains

The American Type Culture Collection (ATCC) reference strains used in our investigation, were *S. pneumoniae* (ATCC 49619), *S. pyogenes* (ATCC 19615), and *Enterococcus faecalis* (ATCC 29212). These organisms were used as control strains. The strains were grown, harvested in exponential growth phase, and fixed with ethanol as described previously [17–19].

Probes

Oligonucleotide probes used for this work (Table I) were synthesized and 5'-labeled with fluorochromes Cy3 or Fluo (Metabion, Martinsried, Germany). The probes Spn [7], Strpyo [17], and Enc [7], which target and hybridize *S. pneumoniae*, *S. pyogenes*, and *Enterococcus* spp., respectively, were used for the detection of the related organisms. The 5' end of these probes were labeled with fluorochrome Cy3, which emits a red color. Probe EUB338, which hybridizes nearly all bacteria [20], was 5'-labeled with fluorochrome Fluo, which exhibits a green color.

Table I

Oligonucleotide probes used in this study

Probe	Sequence (5'–3')	Fluorochrome	Target	Reference
EUB338	GCT GCC TCC CGT AGG AGT	Fluo	Most bacteria	20
Spn	GTG ATG CAA GTG CAC CTT	Cy3	*S. pneumoniae*	7
Strpyo	CTA ACA TGC GTT AGT CTC TC	Cy3	*S. pyogenes*	17
Enc	CCC TCT GAT GGG TAG GTT	Cy3	*Enterococcus* spp.	7

Detection of S. pneumoniae *by FISH*

Detection of *S. pneumoniae* in blood cultures was performed by two FISH procedures: one procedure was carried out with an enzymatic treatment step and the other one was done without enzymatic treatment.

In brief, 10 µl of fixed blood culture specimens or fixed control strains were placed on glass slides which were left to air-dry. The dehydration step was performed in an ascending ethanol series. Enzymatic treatment was carried out with 1 mg/ml lysozyme (Sigma, Steinheim, Germany) for 10 minutes at 30ºC [7]. The hybridization step using two probes EUB338-Fluo and Spn-Cy3 was done at 46ºC for 90 minutes with a hybridization buffer containing 20% formamide described elsewhere [7, 17, 18]. For stringent washing, the slides were incubated in a washing buffer as described previously [17, 18]. Subsequently, DNA was stained with 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI; Roche, Mannheim, Germany).

At the same time, an aliquot of all the 77 blood culture samples and control strain were also tested without enzymatic treatment on separate glass slides, i.e., the procedure was similar to that described above, but the enzymatic treatment step was omitted.

Finally, the slides were observed and analyzed with a Nikon 80i epifluorescence microscope (Nikon, Tokyo, Japan). Microscopy was performed in a blinded manner by two persons.

Detection of S. pyogenes *and* Enterococcus *by FISH*

The blood culture specimens and control strains [*S. pyogenes* (ATCC 19615) and *E. faecalis* (ATCC 29212)] were also examined by the procedure with enzymatic treatment using a mixture of the two probes EUB338-Fluo and Strpyo-Cy3, as well as a mixture of the two probes EUB338-Fluo and Enc-Cy3.

Moreover, *S. pyogenes* (ATCC 19615) and *E. faecalis* (ATCC 29212) were tested by the procedure without enzymatic treatment. Microscopy was done as described above.

Results

Table II shows the results of the examination of 77 blood culture samples for the identification of *S. pneumoniae* by means of conventional culture methods and FISH. By conventional culture, 10 of 77 samples were positive and 67 samples were negative for *S. pneumoniae*. Using FISH with an enzymatic treatment step, *S. pneumoniae* was identified in 9 of 10 culture-positive specimens, whereas without enzymatic treatment, all 10 culture-positive specimens were FISH-positive. All FISH-positive specimens were culture-positive. The FISH results were compared with those of conventional culture methods. Based on the results of this study, the specificity of FISH for the detection of *S. pneumoniae* in blood culture specimens was 100% and the sensitivity of the FISH procedure with enzymatic treatment was 90%, whereas the sensitivity of the FISH procedure without an enzymatic treatment step was 100%.

Results of the examination of *S. pneumoniae* (ATCC 49619) and some *S. pneumoniae*-positive blood cultures showed that omission of enzymatic treatment from the FISH technique led to increasing the intensity of the fluorescence signal of this bacterium; also, by this procedure, the numbers of hybridized *S. pneumoniae* cells were increased.

Table II

Examination of 77 blood culture specimens by FISH (with and without enzymatic treatment) and culture method for the detection of *S. pneumoniae*

Method and result	Number of samples
Culture	
Positive	10
Negative	67
Culture positive	
FISH Positive (with enzymatic treatment)	9
FISH Positive (without enzymatic treatment)	10
FISH Negative (with enzymatic treatment)	1
FISH Negative (without enzymatic treatment)	0
Culture negative	
FISH Positive (with enzymatic treatment)	0
FISH Positive (without enzymatic treatment)	0
FISH Negative (with enzymatic treatment)	67
FISH Negative (without enzymatic treatment)	67

All 77 blood culture specimens were also tested by FISH and culture methods to detect *S. pyogenes* and *Enterococcus*. Since omission of enzymatic treatment impeded detection of the reference strains of *S. pyogenes* and *Enterococcus,* only FISH procedure including enzymatic treatment was carried out for the detection of these microorganisms from blood cultures. In 2 of 77 specimens, *S. pyogenes* was identified by both FISH and cultivation methods. The remaining 75 specimens were negative for this species. Moreover, 4 specimens were *Enterococcus*-positive according to culture and FISH, but 73 specimens were *Enterococcus*-negative according to both methods.

Taken together, when FISH procedure was performed with an enzymatic treatment step for the detection of *S. pneumoniae, S. pyogenes,* and *Enterococcus,* its overall sensitivity was 93.7%, whereas by omission of the enzymatic treatment step during detection of *S. pneumoniae,* this overall sensitivity was 100%. In all conditions, the specificity of FISH was 100%.

Discussion

Application of FISH for the detection of microorganisms within the various specimens has already been reported [7, 17–19, 21]. The cell wall of the Gram-positive bacteria has a thick peptidoglycan layer [22]. In the FISH technique, partial cell wall digestion with lysozyme (e.g. for streptococci) [7, 8, 18] or lysozyme and lysostaphin (for staphylococci) [7] lead to penetrate the oligonucleotide probes into the Gram-positive cells. We designed this investigation to test the FISH protocol without using of lysozyme for the detection of *S. pneumoniae*.

In this study, by means of a modified FISH method (FISH without enzymatic treatment), *S. pneumoniae* was identified in all 10 culture-positive specimens and a 100% sensitivity was achieved. In four *S. pneumoniae*-positive blood culture specimens, there was no difference between the results of FISH procedure with enzymatic treatment and procedure without enzymatic treatment regarding detection of *S. pneumoniae*. However, application of enzymatic treatment produced an adverse effect on the FISH results of five *S. pneumoniae*-positive blood culture specimens, as well as *S. pneumoniae* (ATCC 49619) although these results were not negative; on the other hand, by omission of the enzymatic treatment for examination of these five specimens and control strain, better FISH results were achieved so that the intensity of specific fluorescence signal and/or the number of hybridized *S. pneumoniae* cells were increased and the detection of this bacterium was facilitated and improved. Finally, there was one false-negative result by per-

forming the FISH procedure with the enzymatic treatment step, However, when this step was omitted from the FISH protocol, *S. pneumoniae* was successfully detected. The reason for these results might be the fact that *S. pneumoniae* itself produces lysozyme [23], which can partially digest the cell wall so that oligonucleotide probes can then penetrate into the bacterial cells. Perhaps when an additional treatment with exogenous lysozyme is carried out in the FISH method, digestion of the peptidoglycan can progress more than needed and it can thus cause bacteriolysis so that weak results or even false-negative results for FISH for some specimens can be observed. Taken together, we found that enzymatic treatment was not necessary for the detection of *S. pneumoniae* within the samples tested in our study and even in some cases it could have some adverse effect on the FISH results.

In contrast to our results, in the study conducted by Kempf et al., 7 blood culture specimens were *S. pneumoniae*-positive according to both the conventional culture method and FISH with enzymatic treatment [7]; also, in the study done by Peters and colleagues, *S. pneumoniae* was identified in 2 blood cultures by using both mentioned methods [9]. The reasons for these different results may be due to some factors such as possible dissimilarities in various strains of *S. pneumoniae* for production of lysozyme or differences in enzymatic activity.

Furthermore, we examined the blood cultures by FISH including enzymatic treatment and conventional culture methods for the detection of *S. pyogenes* and genus *Enterococcus*; the results of FISH and culture methods were completely concordant. Similarly, in the investigation conducted by Kempf et al., results of FISH and culture were compatible for the detection of *Enterococcus* [7]. Also, in the study done by Peters et al., results of both mentioned methods were compatible for the identification of *S. pyogenes* [9].

In conclusion, it seems that enzymatic treatment has an adverse effect on FISH results of some strains of *S. pneumoniae* and omission of this step from FISH procedure can facilitate and improve the detection of these strains. Thus, in order to detect *S. pneumoniae,* we suggest the application of the two FISH protocols for each blood culture specimen: one is FISH with enzymatic treatment and the second one is FISH without enzymatic treatment step. A specimen can be reported as *S. pneumoniae*-positive, if this microorganism was detected by at least one of these two protocols. It appears that by this strategy, *S. pneumoniae* can successfully be detected from all blood culture samples containing this organism.

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